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Reversed Alterations of Hippocampal Parvalbumin and Protein Kinase C-γ Immunoreactivity After Stroke in Spontaneously Hypertensive Stroke-Prone Rats

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Background and Purpose: **Aging spontaneously hypertensive stroke-prone rats (SHR-SP) were previously shown to develop neocortical strokes. Because the hippocampal CA1 is selectively vulnerable to abnormal brain perfusion, the neuropathological effects of spontaneous strokes were investigated on specific neurochemical alterations in two major cell types of the hippocampal CA1 in SHR-SP.**

Methods: **The immunoreactivity for the γ-isoform of protein kinase C (in pyramidal cells) and parvalbumin (in interneurons) was determined in the hippocampal CA1 by applying monoclonal antibodies. Because chronic treatment with the calcium antagonist nimodipine prevents the development of strokes in SHR-SP, we compared SHR-SP (stroke) with age-matched nimodipine-treated rats (nonstroke).**

Results: **After stroke in control animals, we observed a strikingly enhanced immunoreactivity for protein kinase C-γ in CA1 pyramidal cells compared with nimodipine-treated rats, which can be interpreted as the result of an increased activation of these cells. The pathological increase of protein kinase C-γ immunoreactivity was accompanied by a reduced parvalbuminergic innervation of these pyramidal cells in symptomatic SHR-SP.**

Conclusions: **Because parvalbumin is present in a subset of GABAergic inhibitory interneurons, these data suggest that increased activity of CA1 pyramidal cells after spontaneous stroke may partially be related to a decreased inhibitory input on these cells. (*Stroke*. 1993;24:2082-2086.)**

KEY WORDS • **hippocampus** • **nimodipine** • **protein kinases** • **rats**

Essential hypertension is a common phenomenon in the elderly[1] and is considered a major risk factor for the occurrence of stroke.[2] The spontaneously hypertensive stroke-prone rat (SHR-SP) strain, obtained by selective breeding of SHR,[3] has proven to be a valuable pathogenetic model for the study of experimental hypertension and stroke.[4] At the age of 43 to 52 weeks SHR-SP develop cerebral hemorrhages and infarcts (strokes), which shortens the mean life span of SHR-SP compared with normotensive Wistar rats.[5,6] Cerebrovascular lesions in SHR-SP predominantly occur in the neocortex at the boundaries between the middle cerebral artery and the anterior and posterior cerebral artery.[3]

Most studies on neurochemical alterations after abnormal perfusion of the brain are based on experimentally induced ischemia (for review, see Reference 7). Ischemia gives rise to neuronal damage, with the pyramidal cells in the hippocampal CA1 being one of the most vulnerable cell types in the central nervous system.[8] The massive release of the excitatory amino acid glutamate in the hippocampus during cerebral ischemia is a key factor in ischemia-induced neuronal damage and cell death.[9] Enhancement of the activity and/or translocation of the enzyme protein kinase C (PKC) might also play an important role in postischemic alteration of neuronal functioning in the hippocampus.[10-12] More recently some attention has also been directed to possible alterations in postsynaptic inhibitory processes after ischemia.[13]

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Much less is known about neurochemical alterations after spontaneous strokes. We previously showed that chronic application of the calcium entry blocker nimodipine starting at the age of 46 weeks prevented the occurrence of stroke in SHR-SP[5,6] without reducing the (high) systolic blood pressure.[5] Comparison of age-matched SHR-SP with (nontreated) and without (nimodipine-treated) spontaneous strokes enabled us to reveal neurochemical alterations that occur after spontaneous strokes. In the present study we examined the effect of stroke in aging SHR-SP on the expression of PKCγ and the calcium binding protein parvalbumin (PV) in dorsal hippocampal pyramidal and nonpyramidal inhibitory interneurons, respectively.

Materials and Methods

The current investigation is based on observations in 22 male SHR-SP (breeder: Møllegaard, Skønevød, Den-

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mark). At the age of 46 weeks the animals were divided into two groups (both n=11), one of which received standard food pellets (SP-placebo). The other group (SP-nimo) received identical food pellets to which 860 ppm nimodipine (Bayer AG, Leverkussen, Germany) was added. Treatment was carried out for a period of 10 weeks (from 46 to 56 weeks), after which the experiment had to be terminated because 4 of 11 placebo animals had died as a result of stroke. The remaining 7 SP-placebo and 7 randomly selected SP-nimo animals were deeply anesthetized with 60 mg/kg IP sodium pentobarbital. Brains were fixed by means of transcardial perfusion with 400 mL fixative containing 3% paraformaldehyde, 0.05% glutaraldehyde, and 2% picric acid in 0.1 mol/L phosphate buffer (PB) at pH 7.4. The brains were removed, and the brain weight of each animal was determined. The brains were stored overnight at 4°C in 30% buffered sucrose for cryoprotection and subsequently cut on a cryostat microtome to 20-μm coronal sections.

For immunocytochemical stainings of PV and PKCγ we used previously described protocols.[14,15] In short, sections from SP-placebo and SP-nimo animals were pooled and incubated overnight at 4°C in phosphate-buffered saline (PBS) containing either monoclonal mouse anti-PV immunoglobulin (Ig) G (Sigma Chemical Co, St Louis, Mo; diluted at 1:2000) or mouse anti-PKCγ IgG (36G9, monoclonal antibody raised against purified bovine PKCγ,[16] kindly donated by Dr S. Cazaubon, Paris, France; diluted at 1:200). Subsequently, the sections were exposed to biotinylated sheep anti-mouse IgG (Amersham, 1:200 in PBS, 2 hours at room temperature) and streptavidin–horseradish peroxidase (Zymed, 1:200 in PBS, 2 hours at room temperature). In both immunostainings, the sections were rinsed at least four times between all incubation steps in PBS, to which in the case of PV 0.5% Triton X-100 was added. Immunolabeling was visualized by 0.03% diaminobenzidine and 0.01% H_2O_2. Standard control experiments were performed by omission of the primary antibodies, yielding absence of any detectable immunolabeling. The brain weight data were tested by analysis of variance (ANOVA), with statistical significance defined as $P<.05$.

Results

During the treatment period (46 to 56 weeks of age), all SP-placebo rats showed neurological symptoms of strokes, such as hyperirritability, motion disturbances, and behavioral depression.[3] After light microscopic analysis of the brain sections, neocortical hemorrhages and infarcts were encountered in all SP-placebo animals (Table). The SP-nimo animals did not show any clear neurological symptoms of stroke. Microscopic evaluation, however, revealed signs of a small stroke in the neocortex of two of seven SP-nimo rats (Table).

The occurrence of stroke in SP-placebo rats was accompanied by severe brain edema formation. The brains of all SP-placebo rats were macroscopically swollen, and the brain weight was extremely high (Table). No signs of brain edema were observed in the SP-nimo group, and the mean brain weight of these animals was normal (Table) and in the same range as that of normotensive controls.[5] Moreover, the brain weight of the two SP-nimo animals in which signs of stroke were found did not differ from the other SP-nimo animals. Statistical analysis showed that the brain weight in the SP-placebo group was significantly higher than in the SP-nimo group (ANOVA: $F_{1,12}=34.403$, $P<.0001$).

Number of Rats, Number of Rats With Histological Signs of Stroke, and Brain Weight of Stroke-Prone Rats in Placebo-Treated and Nimodipine-Treated Groups

Group	n	No. of Rats With Stroke	Brain Weight, g (mean±SEM)
SP-placebo	7	7	2.54±0.11
SP-nimo	7	2	1.81±0.02*

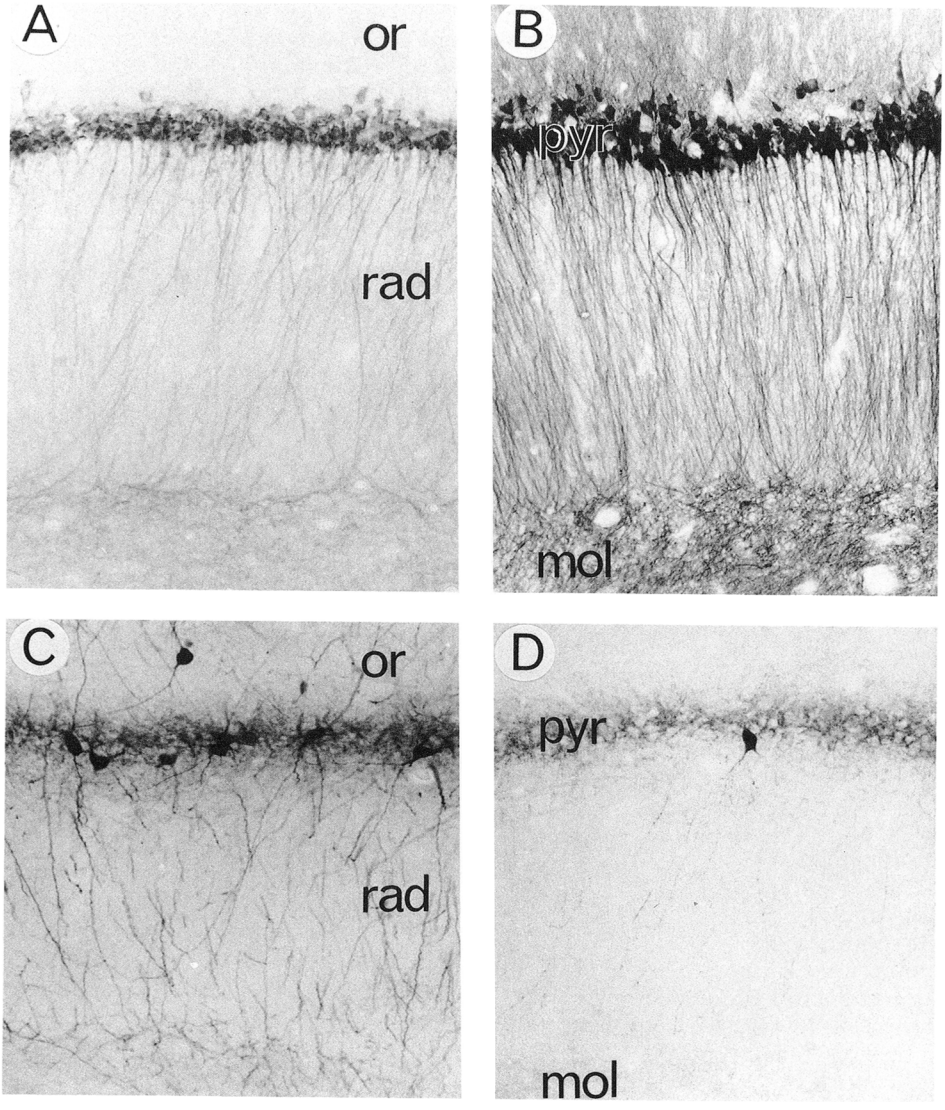
SP-placebo indicates stroke-prone rats in placebo-treated group; SP-nimo, stroke-prone rats in nimodipine-treated group.
*$P<.0001$ (analysis of variance: $F_{1,12}=34.403$).

The cellular distribution and intensity of PKCγ immunoreactivity (ir) in the dorsal hippocampal CA1 of SP-nimo animals (Figure, panel A) were similar to that in normotensive Wistar rats and in mice.[15] In short, moderate levels of PKCγ-ir were found in pyramidal and nonpyramidal cell bodies, and apical dendrites of the CA1 pyramidal cells were also moderately stained. In contrast to the SP-nimo group, PKCγ-ir in all SP-placebo rats showed conspicuous alterations. In large parts of the hippocampus the staining intensity was prominently increased. A much more dense immunostaining was consistently found in the CA1 pyramidal cell layer. Cell bodies, their apical dendrites in the stratum radiatum and lacunosum moleculare, and basal dendrites in the stratum oriens showed an enhanced PKCγ-ir.

In the hippocampal CA1 of SP-nimo rats, the localization of PV-ir (Figure, panel C) closely resembled earlier descriptions in normotensive rats[14,17] and was found in the nonpyramidal cell type. In the CA1 most PV-ir was situated in the stratum pyramidale and stratum oriens. The immunoreactive dendrites extended into the stratum radiatum and stratum oriens and are characterized by their varicose appearance. The PV-ir interneurons gave rise to a dense axonal plexus around the cell bodies and proximal dendrites of the CA1 pyramidal cells. In all SP-placebo animals PV-ir in the CA1 was considerably lower (Figure, panel D). A reduced amount of PV-ir in both cell bodies and dendrites was observed, and the density of the axonal plexus surrounding CA1 pyramidal cells also declined. In the two SP-nimo animals that developed a minor stroke, there were no obvious qualitative alterations in PV- or PKCγ-ir.

Discussion

We examined immunocytochemical alterations in the hippocampal CA1 after spontaneous strokes by comparing aging nontreated SHR-SP with aging, nimodipine-treated SHR-SP. Administration of the L-type calcium channel blocker nimodipine from the age of 46 weeks prevented the occurrence of strokes in 80% of the SHR-SP.[5] Although signs of a minor stroke were observed in two nimodipine-treated rats, the possibility that these animals developed cerebrovascular lesions before the treatment was started cannot be excluded. In the present study we used nimodipine-treated animals as nonstroke controls. In addition to its cerebrovascular



Photomicrographs of the distribution of protein kinase C-γ(PKCγ) (A,B) and parvalbumin (PV) (C,D) in the hippocampal CA1 of control (right panels) and nimodipine-treated (left panels) spontaneously hypertensive stroke-prone rats. Photographs of both PKCγ and PV are taken from the same animal. Note that after stroke (control rat) the immunoreactivity for PKCγ increases in pyramidal cell bodies as well as in apical and basal dendrites. In the same hippocampal region the immunoreactivity for PV decreases. Fewer labeled dendrites and interneurons were observed, and the density of the axosomatic plexus surrounding CA1 pyramidal cells was also reduced. or indicates stratum oriens; rad, stratum radiatum; pyr, stratum pyramidale; and mol, lacunosum moleculare.

actions, nimodipine also directly antagonizes neuronal calcium influx.[18] Recent pilot studies did not reveal alterations in the expression of PKCγ-ir and PV-ir in young SHR-SP treated with nimodipine, which supports the use of SP-nimo animals as nonstroke controls.

In contrast to SP-placebo animals, which all developed strokes and displayed extreme brain edema, the two symptomatic SP-nimo animals did not show any brain tissue swelling. Beneficial effects of nimodipine on the development of edema after experimentally induced ischemia have previously been described.[19] If the strokes in the SP-nimo animals did occur after the treatment was started, nimodipine may have prevented edema formation after spontaneous strokes.

In experimentally induced cerebral ischemia, the pyramidal cells of the hippocampal CA1 are selectively vulnerable and eventually die.[8] Spontaneous strokes did not yield cellular necrosis in the hippocampal CA1 (data not shown); however, prominent neurochemical changes were observed in aging symptomatic SP-placebo rats. In the dorsal hippocampus, CA1 pyramidal cells showed a striking increase of PKCγ-ir, whereas the PV-ir in interneurons was profoundly reduced after stroke. These alterations were consistent and did not correlate with the putative survival time after stroke.

Previously, we showed that PKCγ-ir in pyramidal cells, as visualized with the 36G9 monoclonal antibody, was enhanced in both cell bodies and dendrites after electrical stimulation of the amygdala in a kindling model for epilepsy[20] and chemical stimulation with carbachol and phorbol esters.[15,21] We therefore interpret neurons with enhanced PKCγ-ir as functionally activated cells.[15] Spontaneous cerebrovascular insults and concomitant edema in aging SP-placebo animals were shown in the present study to yield a similar increase in PKCγ-ir in pyramidal cells of the dorsal hippocampal CA1. It has been suggested that PKC activation and translocation play an important role in the pathophysiology of ischemic brain injury and brain edema.[10-12] Using biochemical techniques, Olah et al[22] previously demonstrated a (long-lasting) increased concentration and activity of PKC in the hippocampal CA1 after experimentally induced ischemia in gerbils. Moreover, it was shown that PKC inhibitors can prevent ischemic neuronal damage in hippocampal CA1 neurons.[23] The present data indicate that at least the γ-isoform of PKC shows an enhanced expression after

spontaneous strokes, which corroborates the observations of Wieloch et al,[24] who showed that an increased translocation of PKC in the striatum of ischemic rats is most prominent for the γ-isoform.

The increased pyramidal cell activation, as visualized by an increased PKCγ-ir, after spontaneous strokes may be explained by an increased excitatory input on pyramidal cells, with the neurotransmitter glutamate as the most likely candidate. Numerous reports demonstrate an increased glutamate release in the hippocampus after experimentally induced ischemia, which has been associated with neuronal damage (for review, see Reference 9).

We also observed a clear loss of PV-ir interneurons in the CA1 after spontaneous strokes. PV is a calcium binding protein that is present in a subset of hippocampal γ-aminobutyric acid (GABA)-ergic inhibitory interneurons innervating the hippocampal pyramidal cells.[17] In the hippocampus GABA-mediated inhibition plays an important role in regulating pyramidal cell activity,[25] and recent studies have shown that withdrawal of GABA resulted in hyperexcitability of CA1 pyramidal cells.[26] Our data suggest that the enhanced pyramidal cell activity, as indicated by increased PKCγ-ir, may partially be related to a reduced PV/GABAergic inhibitory input on these cells. This option is further substantiated by data from Lyden and Hedges,[13] who showed that administration of the GABA agonist muscimol was equally as protective as the glutamate antagonist MK-801 in ischemia-induced brain damage. Whether the reduced amount of PV-ir interneurons after spontaneous stroke is related to GABAergic cell loss is under current investigation.

In summary, the present study shows that the occurrence of spontaneous strokes and accompanying edema formation in aging SHR-SP coincides with consistent alterations of neuronal functioning in the hippocampal CA1. The activity of CA1 pyramidal cells is increased, which may partially be related to a decreased inhibitory input on these cells. Because CA1 pyramidal cells are the major output cells of the hippocampus, hyperactivity of these cells is likely to disturb hippocampus-mediated functions, such as adequate cognitive performance in learning and memory tasks.

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